Untargeted metabolomics profiling of skeletal muscle samples from malignant hyperthermia susceptible patients

Profilage métabolomique non ciblé d'échantillons de muscles squelettiques de patients susceptibles à l'hyperthermie maligne

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Abstract

Purpose Malignant hyperthermia (MH) is a potentially fatal hypermetabolic condition triggered by certain anesthetics and caused by defective calcium homeostasis in skeletal muscle cells. Recent evidence has revealed impairment of various biochemical pathways in MH-susceptible patients in the absence of anesthetics. We hypothesized that clinical differences between MH-susceptible and control individuals are reflected in measurable differences in myoplasmic metabolites.

Barbara Bojko and Tijana Vasiljevic have contributed equally to this work.

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Methods We performed metabolomic profiling of skeletal muscle samples from MH-negative (control) individuals and MH-susceptible patients undergoing muscle biopsy for diagnosis of MH susceptibility. Cellular metabolites were extracted from 33 fresh and 87 frozen human muscle samples using solid phase microextraction and Metabolon® untargeted biochemical profiling platforms, respectively. Ultra-performance liquid chromatography-high resolution mass spectrometry was used for metabolite identification and validation, followed by analysis of differences in metabolites between the MH-susceptible and MH-negative groups.

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Results Significant fold-change differences between the MH-susceptible and control groups in metabolites from various pathways were found (*P* value range: 0.009 to < 0.001). These included accumulation of long chain acylcarnitines, diacylglycerols, phosphoenolpyruvate, histidine pathway metabolites, lysophosphatidylcholine, oxidative stress markers, and phosphoinositols, as well as decreased levels of monoacylglycerols. The results from both analytical platforms were in agreement.

Conclusion This metabolomics study indicates a shift from utilization of carbohydrates towards lipids for energy production in MH-susceptible individuals. This shift may result in inefficiency of beta-oxidation, and increased muscle protein turnover, oxidative stress, and/or lysophosphatidylcholine levels.

Résumé

Objectif L'hyperthermie maligne (HM) est une condition hypermétabolique potentiellement mortelle déclenchée par certains agents anesthésiques et causée par une homéostasie calcique perturbée des cellules musculaires squelettiques. Des données probantes récentes ont mis en lumière une atteinte de diverses voies biochimiques chez les patients susceptibles à l'HM en l'absence d'anesthésiques. Nous avons émis l'hypothèse que les différences cliniques entre les individus susceptibles à l'HM et des témoins se refléteraient dans des différences mesurables de métabolites myoplasmiques.

Méthode Nous avons réalisé un profilage métabolomique d'échantillons de muscles squelettiques provenant de personnes négatives à l'HM (témoins) et de patients susceptibles à l'HM subissant une biopsie musculaire dans le but de poser un diagnostic de susceptibilité à l'HM. Les métabolites cellulaires ont été extraits de 33 échantillons de muscles humains frais et de 87 échantillons congelés à l'aide d'une microextraction en phase solide et des plateformes de profilage biochimique non ciblées Metabolon®, respectivement. La chromatographie en phase liquide à haute performance et la spectrométrie de masse à haute résolution ont été utilisées pour l'identification et la validation des métabolites, puis suivies d'une analyse des différences

dans les métabolites entre les groupes susceptibles à l'HM et les groupes négatifs à l'HM.

Résultats Des différences significatives ont été observées entre les groupes susceptibles à l'HM et les groupes témoins dans les métabolites issus de diverses voies (*P* : de 0,009 à < 0,001). Ces différences comprenaient l'accumulation d'acylcarnitines à longue chaîne, de diacylglycérols, de phosphoénolpyruvate, de métabolites de la voie d'histidine, de lysophosphatidylcholine, de marqueurs de stress oxydatif, et de phosphoinositols, aussi bien que des taux réduits de monoacylglycérols. Les résultats des deux plateformes analytiques concordaient.

Conclusion Cette étude métabolomique indique un changement de l'utilisation des glucides vers les lipides pour la production d'énergie chez les personnes susceptibles à l'HM. Ce changement pourrait entraîner une inefficacité de la bêta-oxydation, ainsi qu'une augmentation du renouvellement des protéines musculaires, du stress oxydatif, et/ou des taux de lysophosphatidylcholine.

Keywords Metabolomics · Malignant hyperthermia

Malignant hyperthermia (MH) is a potentially fatal pharmacogenetic disorder of skeletal muscle, triggered by exposure to certain anesthetics.¹ During an MH reaction, there is a sustained elevation in myoplasmic calcium (Ca^{2+}) that leads to a hypermetabolic state.^{1,2} The muscle contracture test is a highly sensitive diagnostic test for MH susceptibility³ that requires a freshly-excised muscle to measure the contracture response to caffeine and halothane. The diagnosis of MH susceptibility may also be determined by identifying a pathogenic variant in one of the three MH-related genes—*RYR1*, *CACNA1S*, and *STAC3*^{1,2}—whose products play key roles in the process of excitation-contraction coupling and in maintenance of Ca^{2+} homeostasis in skeletal muscle.

In the absence of anesthetic triggers, about 50% of MH-susceptible (MHS) individuals⁴ present with musculoskeletal symptoms such as myalgia, cramps, heat sensitivity, and exertional rhabdomyolysis. Metabolic changes caused by defective excitation-contraction coupling¹ in MHS individuals have long-lasting effects on skeletal muscle function that have been the subject of recent research. One study hinted at the increased oxidative stress as an underlying pathomechanism.⁵ Furthermore, an *in vivo* metabolic assessment using magnetic resonance spectroscopy in MHS individuals showed an impaired aerobic metabolic pathway with reduced adenosine triphosphate (ATP) production.⁶ Another study using

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permeabilized skeletal muscle cells from MHS individuals showed mitochondrial dysfunction at baseline.⁷ Some older studies showed a connection between Ca^{2+} dyshomeostasis and impaired fatty acid metabolism in MHS individuals.^{8–10}

Through a novel holistic approach, we aimed to identify pre-existing impairments in the metabolic pathways underlying the MHS phenotype in the absence of anesthetic triggers. We hypothesized that clinically observed differences between MHS and MH-negative (MHN) individuals are reflected in measurable differences in myoplasmic metabolites. Furthermore, by identifying the constellation of biochemical markers peculiar to the MHS phenotype, we should be able to elucidate up-regulated or down-regulated metabolic pathways involved in MH susceptibility that could help identify potential drug targets for both MH prevention and treatment.

Methods

Patients

Following institutional research ethics board approval (13-7116-BE, approved on 7 February 2014), all individuals referred to the Malignant Hyperthermia Investigation Unit at University Health Network between January 2015 and December 2018 for a caffeine halothane contracture test (CHCT)³ were approached for this study. To avoid confounding factors, only individuals with self or family history of an MH reaction (without any other known medical conditions requiring medication) were enrolled in this study. Written informed consent was obtained from all participating individuals.

Muscle sample collection

The muscle biopsy and CHCT were done according to the North American Protocol.³ The excess of biopsied specimen was utilized for metabolomics analysis. We explored two approaches for high-throughput detection and quantification of cellular metabolites: 1) solid phase microextraction (SPME)—a solvent-free, single-step, minimally invasive sampling technique allowing concomitant sample extraction and quenching of the metabolic processes in *fresh muscle tissues*,^{11–14} and 2) an untargeted biochemical profiling procedure developed by Metabolon® (Durham, NC, USA).¹⁵ Both approaches use liquid chromatography-high resolution mass spectrometry that ensures data reproducibility and accurate metabolite identification.

Muscle sample preparation

Muscle biopsy samples were transported to the laboratory in carbogenated Krebs-Ringer buffer because it was technically difficult to dissect them in the operating room. Upon arrival to the laboratory, which on average was five minutes after harvest, muscle strips, dissected from the biopsied muscle tissue and measuring approximately 25 x 5 x 5 mm (~150 mg), were either immediately flash-frozen in liquid nitrogen and shipped on dry ice to Metabolon or used as fresh muscle for sampling with SPME fibres.

Solid phase microextraction sampling and processing

Three 7-mm mix-mode coating SPME fibres were preconditioned in 1:1 methanol:water and then rinsed with water. The SPME fibres were inserted for 15 min into a fresh muscle strip immersed in Krebs-Ringer solution. After sampling was completed, the fibres were removed, washed with water, packed individually in vials and stored at -80°C until analysis. The fibres were removed from -80°C and desorbed to 200 μL 1:1 acetonitrile:water for one hour on a vortex (1,500 rounds per minute agitation). A pooled matrix sample (generated by taking a small volume of each experimental sample), extracted water samples (process blanks), and a cocktail of different quality control standards were analyzed in parallel as controls. Instrument variability was determined by calculating the percent relative standard deviation (RSD) of the instrumental quality controls, injected after every tenth sample. The overall process variability was determined by calculating the percent RSD for all endogenous metabolites present in 100% of the pooled matrix samples. The extracted samples were analyzed in positive and negative ionization modes in random order on a liquid chromatography-mass spectrometry (LC-MS) system consisting of a binary pump, an Accela autosampler, and an Orbitrap mass spectrometer Q-Exactive equipped with a heated electrospray source (Thermo Fischer Scientific, Waltham, MA, USA). Chromatographic separation was carried out as described before.¹⁶

To confirm metabolite identity, significant compounds were analyzed under parallel reaction monitoring conditions via LC-MS using a Q-Exactive Quadrupole Orbitrap (Thermo Fischer ScientificTM, Waltham, MA, USA) and a Dionex Ultimate 3000 UPLC system (Dionex Corporation, Bannockburn, IL, USA).

Metabolon® sampling and processing

The frozen muscle samples were prepared for analysis using the automated MicroLab STAR® system (Hamilton

Company, Reno, NV, USA). The extract was divided into four fractions: two fractions for analysis by two separate reverse phase ultra-performance LC-tandem MS (RP/UPLC-MS/MS) methods with positive and negative ion mode electrospray ionization—one fraction for analysis by hydrophilic interaction chromatography/ultra-performance liquid chromatography-tandem mass spectrometry (HILIC/UPLC-MS/MS) with negative ion mode electrospray ionization, with the other fraction reserved for backup.¹⁷ All methods utilized the Waters ACQUITY ultra-performance liquid chromatography (Waters Corporation, Milford, MA, USA) and a Thermo Scientific Q-Exactive high resolution/accurate mass spectrometer interfaced with a heated electrospray ionization source and Orbitrap mass analyzer operated at 35,000 mass resolution. Full details concerning the LC gradient and MS conditions have previously been described.¹⁸ Quality control was similar to SPME processing as described above.

Data extraction, compound identification, and statistical analysis

The SPME data were obtained in raw format using Thermo Xcalibur software (Thermo Fischer ScientificTM, Waltham, MA, USA) and converted using MSconvert.¹⁹ After conversion, the data were analyzed using R-statistical package (R Foundation for Statistical Computing, Vienna, Austria)²⁰ to remove features whose RSD in the pooled quality controls was > 30% (acceptance criterion proposed by Want *et al.*)²¹ as well as features whose signal-to-noise (S/N) ratio was < 5 compared with pooled quality controls. Further data analysis and normalization were done using MetaboAnalyst software,²² and autoscaling.²³ Data clustering and potential outliers were examined using principal component analysis and partial least squares discriminant analysis (PLS-DA). Details of validation of the PLS-DA model and identification of statistically significant features/metabolites using variable importance in projection (VIP) are provided in the Electronic Supplementary Material (ESM; eTables 1 and 2). Non-parametric tests (Wilcoxon's rank *t* test, unequal group variance) based on a two-sided hypothesis were applied for the identification of significant metabolites with a significant *P* value set at < 0.05, and false discovery rate (FDR) based on Benjamini-Hochberg adjustment set at 5%.

Identification of metabolites was done by comparing their exact masses obtained from LC-MS with the Metlin²⁴ or human metabolome database (HMDB),²⁵ or from the Metabolon library of authenticated standards. Two-step confirmations were performed after determining the potential metabolites. The first step was to run both standards and samples under the previous chromatographic conditions, and the obtained retention time of the feature

was compared with the standard's retention time. The fragmentation patterns of the standard and the metabolite were compared and used as second confirmation. In absence of standards (for SPME samples), the fragmentation patterns were compared with patterns reported in the Metlin database or HMDB (Fig. 1).

For Metabolon samples, standard statistical analyses were performed on log-transformed data. Welch's two-sample independent *t* test was used for a two-sided hypothesis of whether the metabolite means were different between the two study groups. To correct for false discovery of statistically significant compounds due to multiple hypothesis testing, Benjamini-Hochberg adjustment method with a FDR (*q* value) of < 5% was used. Fold differences were determined by dividing relative abundance of a metabolite in the MHS samples by its relative abundance in the MHN samples. Metabolites with *P* < 0.05 and *q* < 0.05 were considered statistically significant in this study. Because of lack of previous data, a statistical power calculation was not conducted for either SPME or Metabolon approach. The sample size was based on the available study data. Both methods used *post hoc* assignment of biochemical pathways. Methodological details for both methods have been previously described.^{18,26,27}

Results

Based on the inclusion criteria, muscle biopsy samples from 14 MHS and 19 MHN individuals obtained in 2015 and 2017 were analyzed by SPME technology; samples from 54 MHS and 33 MHN individuals obtained between 2015 and 2018 were analyzed by Metabolon technology. Nine MHS and five MHN samples were analyzed by both methods. Data from all recruited patients were included. The details of patients' demographics are listed in Table 1.

Solid phase microextraction results

For the 2015 cohort, a total of 15,945 and 4,985 features (a peak or signal that represents a chemical compound) were obtained in positive and negative ionization modes, respectively. Results from the negative ionization mode, however, were excluded because the detected features were assessed as insignificant/non-informative using MetaboAnalyst. The 2017 cohort yielded 10,973 and 5,871 features for positive and negative ionization modes, respectively. Good clustering of data were obtained for both cohorts using partial least squares discriminant analysis (PLS-DA) (Fig. 2). The PLS-DA²⁸ models were validated by examining the results of a ten-fold cross validation and using Q^2 as a performance measure whereby

Metabolon® approach

SPME approach

Fig. 1 Steps from sampling to analysis for Metabolon® (upper panel) and for SPME (lower panel) techniques. Picture shows a sample of muscle inserted with SPME fibres. QC = quality control; SPME = solid phase micro-extraction; (RP)UPLC MS/MS = reverse phase ultra-performance liquid chromatography-tandem mass spectrometry

TABLE 1 Characteristics of the recruited patients

Patient group	Number of patients (<i>n</i>)	Males (<i>n</i>)	Age mean (SD)	Caffeine contracture, g Median (range)	Halothane contracture, g Median (range)	Genetics		
						Negative (<i>n</i>)	Positive (<i>n</i>)	
							Pathogenic	VUS*
SPME group								
MHS	14	8	35.5 (12)	0.3 (0–3.4)	1.6 (1–10.6)	4	0	4
MHN	19	6	38.9 (8.8)	0.0 (0–0.2)	0.3 (0–0.6)	19	0	0
Metabolon® group								
MHS	54	28	34.5(13.9)	0.7 (0–8.6)	2.8 (1–12.5)	16	18	20
MHN	33	15	35.5(13.8)	0.0 (0–0.2)	0.4 (0–0.6)	33	0	0

g = gram contracture; MHN = malignant hyperthermia negative; MHS = malignant hyperthermia susceptible; SD = standard deviation; SPME = solid phase microextraction; *VUS = variant of uncertain significance. RYR1 and CACNA1S genes were fully sequenced in both MHS and MHN groups

the 2015 cohort yielded acceptable results ($R^2 = 0.84$, $Q^2 = 0.56$; see ESM, eTable 1), while 2017 cohort data were found to be over-fitted (ESM, eTable 1).

Additionally, we performed a permutation test ($n = 2,000$) with separation distance (B/W), which revealed that the PLS-DA model of the 2015 cohort gave a test statistics of 0.06, while the 2017 cohort's PLS-DA showed P values of 0.99 and 0.14 for positive and negative ionization mode, respectively (ESM, eTable 2). Further evaluation using non-parametric Wilcoxon's rank t test with FDR < 0.05

yielded a total of 829 significant compounds with 108 being up-regulated in MHS cases for the 2015 cohort. Tentatively identified metabolites corresponded to acylcarnitines, vitamin D metabolites, long chain fatty alcohols, fatty acids, prostaglandins, amino acids, and selected lipid species. A single metabolite (16:0 lysophosphatidylcholine) was validated, further suggesting involvement of lipid species in MH susceptibility. Evaluation of the 2017 cohort revealed a limited number of significant features when using a non-

Fig. 2 Partial least squares discriminant analysis (PLS-DA) plots of “2015” cohort in positive ionization mode (A), “2017” cohort in positive ionization mode and negative ionization mode (B and C).

The components were selected to show the optimum separation in two-dimensional view

parametric analysis of variance test, so heatmaps were used to determine features of potential interest according to their VIP scores. Data representing the involved pathways and statistically significant metabolites with highest or lowest fold changes for both cohorts can be seen in Table 2. (Detailed statistical information is included in the ESM eTable 3).

Metabolon results

The data set includes 498 compounds of known identity—i.e., metabolites detected in skeletal muscle samples. Following log transformation and imputation of missing values, with the minimum observed value for each compound, Welch’s two-sample *t* test identified metabolites that differed significantly between MHS and MHN experimental groups. Based on an FDR < 0.05, a total of 94 metabolites were significantly different between MHS and MHN, of which 67 metabolites were significantly increased and 27 significantly decreased in the MHS group compared with in the MHN group.

Table 3 shows four involved pathways—lipid, amino acid, carbohydrate, and glutathione—represented by some of the validated statistically significant metabolites with the highest and the lowest fold changes between the two groups. Consistent with SPME findings, the results indicate accumulation of medium and long chain fatty acids, and increased levels of phosphatidylcholines, protein degradation products, and oxidative stress indicators.

Discussion

Comparative analysis of muscle samples from MHS and MHN patients revealed significant differences in levels of

metabolites involved mainly in lipid, carbohydrate, and glutathione metabolism. Both SPME and Metabolon analysis revealed a similar pattern of metabolite differences between the two patient groups. Solid phase microextraction was performed on a fresh muscle sample, yielding a large number of metabolites. Nevertheless, the validation of metabolites identified by Metabolon was more comprehensive.

One of the major findings in the MHS group was increased levels of polyunsaturated fatty acids, and reduced levels of several monoacylglycerols. Free fatty acids can be derived from lipolysis of triacylglycerols, and the rate of fatty acid β -oxidation is regulated by the availability of coenzyme A and by the carnitine shuttle. Elevated levels of free fatty acids in the MHS group may indicate that they are “primed” for ATP generation from lipid oxidation. Lower levels of monoglycerols indicate increased lipolysis, resulting in the elevated levels of polyunsaturated fatty acids. These results point towards an increased lipid utilization for energy production. The shift of energy production to lipids from carbohydrate sources is also inferred from the accumulation of phosphoenolpyruvate along with reduced levels of other intermediates such as glucuronate, alpha ketoglutarate, and isocitrate, which are indicators of an impaired Krebs cycle. The excessive accumulation of acylcarnitines can result from inefficient importation of fatty acids by “overwhelmed mitochondria” (i.e., inefficiency of lipolysis pathway due to overuse). Our findings are in agreement with previous studies in animals and with our own *in vivo* metabolic study.^{6,29–34}

Significant increases in oxidized glutathione, cysteinylglycine, 5-oxoproline, and opthalmate found in MHS muscle, consistent with previous studies, indicate impaired antioxidant utilization and increased oxidative stress associated with MH susceptibility.^{32,33,35}

TABLE 2 Solid phase microextraction results

Pathway	Metabolite(s)	MHS/ MHN RSD (%)	<i>P</i> value
I-Lipid metabolism			
Fatty acid metabolism (acyl carnitines)	Alpha-linoeoyl carnitine	2.50 (22.9)	0.003
	Pentadecanoylcarnitine	2.38 (11.2)	0.004
	Tiglylcarnitine	5.36 (10.5)	<0.001
Fatty acid metabolism	Octadecanol	2.58 (11.5)	0.002
	(20R,22R)-20,22-hydroxycholesterol	2.72 (10.6)	0.001
	5-tetradecenoic acid	2.17** (18.5)	0.001
	3-methylglutaconic acid	0.76** (9.3)	<0.001
	(±)19,20-Dihydroxy-4Z,7Z,10Z,13Z,16Z-docosapentaenoic acid	2.94 (11.3)	<0.001
Fatty acid amides	N-palmitoylethanolamide	1.91** (21.1)	0.003
Polyunsaturated fatty acids	Arachidonic acid	2.75** (11.4)	<0.001
	Linoleic acid	1.18* (8.2)	<0.001
	Gamma-linoleic acid	1.13** (5.9)	<0.001
Phospholipid	Phosphatidylcholines*	2.58–6.70 (25.3)	<0.001
	Phosphatidylethanolamines*	2.50–6.71 (19.8)	<0.001
	Phosphatidylserines*	6.71 (6.5)	<0.001
	Phosphatidic acids*	2.54–6.34 (15.9)	<0.001
	Lysophosphatidylethanolamines*	2.57 (10.4)	0.002
	16:0 LysoPC	2.65 (20.36)	0.002
	Phosphatidylinositols*	2.67 – 2.80 (24)	0.01
	Phosphatidylglycerols*	2.36 (15.8)	0.004
	LPA (0:0/18:0)	3.64 (24.3)	<0.001
	PA (18:0/0:0)		
	PA(P20:0/22:6(4Z,7Z,10Z,13Z,16Z,19Z))	3.31 (16.7)	<0.001

TABLE 2 continued

Pathway	Metabolite(s)	MHS/ MHNRS (%)	<i>P</i> value
Triglycerides	Triglycerides*	3.80 (18.7)	<0.001
Mitochondrial beta-oxidation of long chain saturated fatty acids	12-[[[(9Z)-1-oxo-9-hexadecen-1-yl]oxy]-octadecanoic acid 9-[[[(9Z)-1-oxo-9-hexadecen-1-yl]oxy]-octadecanoic acid	2.90 (18.5)	0.001
Acylglycines	Pristanoyl-glycine	0.86** (6.5)	0.012
	Pentadecanoylglycine	0.53** (9.8)	0.017
	4-aminohippuric acid	1.44** (8.4)	<0.001
	Hydroxyvaleryl-glycine	1.64** (21.2)	0.002
Eicosanoids	Leukotrienes*	5.12 (11.1)	<0.001
	15-(R)-15-methylprostaglandin A2 Prostaglandin A2 methyl ester	3.22 (25.3)	<0.001
	16,16-dimethyl-Prostaglandin A2	2.94 (6.2)	0.001
	1a,1b-dihomo-15-deoxy- δ -12,14-Prostaglandin D2	2.51 (10.7)	0.003
	Prostaglandins*	2.85 (18.5)	0.001
2-Amino acid metabolism			
Histidine metabolism	L-histidine	1.28** (15.2)	<0.001
Aminoacyl-t-RNA biosynthesis	N-methyl-proline L-lysine 4-amino-3-hydroxybutyrate L-threonine L-homoserine	1.33** (10.4) 0.24** (3.6)	0.001 0.002
Dipeptide	Asparaginyl-proline Prolyl-asparagine	1.20** (28.7)	0.006
3-Vitamin D metabolism	3-Deoxyvitamin-d3	2.03 (9.5)	0.009
	Vitamin D and derivatives*	2.43–5.12 (19.1)	0.003
	18-acetoxy-1 α ,25-dihydroxyvitamin D3	3.59 (17.9)	<0.001
	3-Deoxy-3-azido-25-hydroxyvitamin D3	3.42 (15.5)	0.02
	24-hydroxycalcitriol	2.54 (19.6)	0.002

Highlights of the involved pathways with fold difference of significant metabolites

*A large number of different hits returned for one or more m/z (mass to charge ratio) values

** VIP score (measure of variable's importance) obtained from a partial least squares discriminant analysis (PLS-DA) plot. MHN = malignant hyperthermia negative; MHS = malignant hyperthermia susceptible; RSD = relative standard deviation

TABLE 3 Metabolon results: highlights of the involved pathways with fold difference of validated significant *metabolites*

Pathway	Metabolite	MHS/MHN Mean (SD)	P value
1-Lipid metabolism			
Fatty acid metabolism (acyl carnitine)	Laurylcarnitine (C12)	3.92 (0.65)	<0.001
	Myristoylcarnitine (C14)	4.29 (0.31)	0.008
	Palmitoylcarnitine (C16)	4.61 (0.91)	0.002
	Palmitoleoylcarnitine (C16:1)*	3.34 (0.28)	<0.001
	Stearoylcarnitine (C18)	4.23 (0.53)	<0.001
	Linoleoylcarnitine (C18:2)*	4.52 (0.32)	<0.001
	Oleoylcarnitine (C18:1)*	3.44 (0.74)	<0.001
Polyunsaturated fatty acid	Arachidonate (20:4n6)	3.98 (0.34)	<0.001
	Dihomo-linolenate (20:3n3 or n6)*	3.17 (0.56)	0.003
	Hexadecatrienoate (16:3n3)	2.87 (0.64)	0.003
Fatty acid synthesis	Malonylcarnitine	4.38 (0.41)	<0.001
Monoacylglycerol	1-oleoyl-GPG (18:1)	0.76 (0.08)	0.004
	1-arachidonoyl-GPI (20:4)	0.85 (0.02)	<0.001
	1-stearoyl-GPC (18:0)	0.70 (0.13)	0.001
	1-palmitoyl-GPC (16:0)	0.64 (0.05)	<0.001
Diacylglycerols	Stearoyl-linoleoyl-glycerol (18:0/18:2) [2]	3.21 (0.37)	<0.001
	Oleoyl-linoleoyl-glycerol (18:1/18:2)	3.93 (0.63)	0.002
	Oleoyl-linolenoyl-glycerol (18:1/18:3)	3.06 (0.58)	0.009
	Oleoyl-arachidonoyl-glycerol (18:1/20:4)	3.23 (0.92)	0.006
	Palmitoleoyl-oleoyl-glycerol (16:1/18:1) [2]	4.35 (0.45)	0.002
	Palmitoyl-linoleoyl-glycerol (16:0/18:2) [2]	3.89 (0.84)	<0.001
Phospholipid	1-16:0-Lysophosphatidylcholine*	4.92 (0.38)	<0.001
	1-palmitoyl-2-docosahexaenoyl-GPC (16:0/22:6)	3.20 (0.95)	0.004
	1-palmitoyl-2-palmitoleoyl-GPC (16:0/16:1)*	2.84 (0.27)	<0.001
2-Amino acid metabolism			
Histidine metabolism	Histidine*	3.39 (0.59)	0.008
	1-methylhistidine	5.64 (0.37)	<0.001
	Carnosine*	4.49 (0.63)	<0.001
	3-Methylhistidine	5.81 (0.79)	<0.001
Dipeptide	Glycylvaline	4.27 (0.77)	0.001
	Isoleucylglycine	4.89 (0.51)	0.009
	Leucylglycine	4.03 (0.18)	0.002
	Phenylalanylglycine*	4.25 (0.85)	0.002
	Prolylglycine	4.43 (0.49)	<0.001
	Valylglycine	4.01 (0.29)	0.003
N-Acetylated amino acids	N-acetyl glycine	0.85 (0.01)	<0.001
	N-acetyl aspartate	0.74 (0.04)	0.004
	N-acetyl serine	0.78 (0.09)	<0.001
	N-acetyl glutamate	0.70 (0.12)	0.005
	N-acetyl methionine	0.82 (0.10)	0.001
3-Carbohydrate metabolism	Phosphoenolpyruvate	2.08 (0.37)	0.002
	Glucurante	0.71 (0.14)	0.001
	Alpha ketoglutarate	0.68 (0.06)	0.002
	Isocitrate	0.72 (0.12)	0.007
4-Glutathione metabolism	Glutathione, oxidized(GSSG)	5.02 (0.44)	0.003

TABLE 3 continued

Pathway	Metabolite	MHS/ MHN Mean (SD)	<i>P</i> value
	Cysteinylglycine	4.71 (0.80)	<0.001
	5-oxoproline	3.85 (0.41)	<0.001
	Ophthalmate	3.54 (0.37)	<0.001

* Indicates metabolites that were significantly increased in nine MHS patients whose muscle samples were analyzed by both solid phase microextraction and Metabolon® approaches. MHS = malignant hyperthermia susceptible

Cysteinylglycine and 5-oxoproline are glutathione catabolites generated when glutathione is recycled in the cell. Ophthalmate is a compositional derivative of glutathione, and its synthesis increases with increased oxidative stress.³⁶

Approximately six-fold increased levels of 3-methylhistidine (an index of the rate of muscle protein breakdown), was present in the MHS compared with the MHN group. Elevated levels of histidine pathway metabolites as observed in the MHS group are suggestive of greater muscle activity and turnover.

The increased levels of diacylglycerol found in the MHS group is consistent with a recent report³⁷ of a higher prevalence of hyperglycemia in MHS patients, as elevated diacylglycerol levels are involved in the development of insulin resistance.³⁸ Diacylglycerols can activate TRPC3 and TRPC6 channels. These same channels were recently shown to be responsible for the increased extracellular Ca^{2+} influx in an MH mouse model.³⁹ Recent evidence also showed that wide-ranging metabolic alterations in MHS individuals stimulate glycogen breakdown and may hamper glucose uptake and utilization inside muscle, thus promoting hyperglycemia in MHS individuals.⁴⁰

One of the validated findings on SPME was an increase in 16:0 lysophosphatidylcholine, a product of hydrolysis of phosphatidylcholine catalyzed by the enzyme phospholipase A₂ (PLA₂). Increased phospholipase A₂ activity results in higher levels of lysophosphatidylcholines and lysophosphatidylethanolamines,⁴¹ possibly due to increased free fatty acid release from mitochondria in MHS patients.^{8–10,42} Lysophosphatidylcholine is also known to be an effector of fatty acid-induced insulin resistance,⁴³ a condition that seem to be more prevalent in MHS patients.^{37,40}

Phospholipase A₂, regulated by phosphorylation and Ca^{2+} ,⁴⁴ plays a role in formation of reactive oxygen species, influencing contractile properties and fatigue characteristics of skeletal muscles.^{41,42,45} In particular, the phospholipase A₂-dependent increase in long chain fatty acid(s) increased the release of Ca^{2+} from skeletal muscle mitochondria in a porcine MH model.⁴⁶ Thus,

increased activity of phospholipase A₂ resulting in increased lysophosphatidylcholine in MHS patients may contribute to increased levels of fatty acids and increased oxidative stress, according to our findings using both approaches.

Other features pointed to several different families of lipids, with strictly returned hits for phosphoinositols. This finding corroborates results of a previous study,⁴⁷ which showed higher basal levels of inositol phosphate products (over 13-fold increase) in MHS swine compared with MHN swine, confirming a correlation between increased levels of phosphoinositols and MH susceptibility.

Our study has some limitations. First, we limited our recruitment to patients with no major morbidities to minimize potential effects of unrelated conditions and their medications on metabolite profiles; however, this decision affected our sample size. Second, MH is a rare disorder, so it is challenging to obtain a very large patient cohort to minimize the confounding effects. Third, our fresh sampling technique (SPME) did not go through full validation of compounds, but results from Metabolon have been fully validated. Fourth, although muscle samples were kept in oxygenated buffer during preparation, the possibility of ischemia and its differential effects between genotypes cannot be excluded. Despite these limitations, the results of our metabolomics profiling of MH susceptibility are encouraging, providing preliminary data on metabolic pathway alterations in MHS individuals in the absence of anesthesia.

The prominence given to MH crises triggered by anesthetics has obscured the fact that MH susceptibility is a pleomorphic condition, manifested with various abnormal phenotypes in the absence of anesthetic triggers. Our holistic approach using metabolomics technology shows up- and down-regulation of various biochemical pathways in MHS patients in the absence of anesthesia, showing their underlying abnormal metabolism. Overall, our findings of faster protein turnover and increased oxidative stress in MHS may indicate fragile muscles with lower threshold to rhabdomyolysis in response to various environmental triggers. The varied

degrees of impairment of the affected pathways may also account for the variable clinical spectrum of MHS—from patients with no symptoms to those complaining of fatigue, heat intolerance, muscle cramps, myalgia, and rhabdomyolysis. These abnormalities have important clinical and pharmacologic implications for the anesthetic management of MHS patients beyond the risk of MH reaction.

In summary, our metabolomics study on MHS individuals showed a high muscle protein turnover as per increased levels of histidine pathway metabolites, increased oxidative stress, and a shift from carbohydrates towards lipids for energy production, which might reduce efficiency of the beta-oxidation pathway, corroborating previous evidence of mitochondrial involvement as a secondary effect of skeletal muscle Ca^{2+} dyshomeostasis. Taken together, these findings reflect metabolic impairment of MHS skeletal muscle in the absence of anesthetics.

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Author contributions Barbara Bojko and Tijana Vasiljevic participated in research conceptualization and design, data interpretation, and drafting of the manuscript. Ezel Boyaci and Anna Roszkowska participated in performing the experiments, data acquisition and interpretation, and drafting of the manuscript. Natalia Kraeva participated in obtaining consent, facilitation of experimental set up, and drafting of the manuscript. Carlos Ibarra Moreno and Annabel Koivu participated in data interpretation, analysis, and drafting of the manuscript. Marcin Wasowicz participated in conception and initial design of the study, and writing and drafting of the manuscript. Amy Hanna participated in data acquisition and drafting of the manuscript. Susan Hamilton participated in research conceptualization and design, and drafting of the manuscript. Sheila Riazzi participated in research conceptualization and design, experimental set up, data interpretation, and drafting of the manuscript. Janusz Pawliszyn participated in research conceptualization and design, supervision of SPME experimental set up, data interpretation, and drafting of the manuscript.

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